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Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains

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ABSTRACT

Rice is the staple food of half of the world's population; however, it is a poor source of essential micronutrients such as Fe and Zn. Since flag leaves are one of the sources of remobilized metals for developing seeds, the identification of the molecular players that might contribute to the process of metal transport from flag leaves to the seeds may be useful for biofortification purposes. We analyzed the expression of 25 metal-related genes from rice, including rice homologues for *YSLs*, *NRAMPs*, *ZIPs*, *IRT1*, *VIT1* (coding for known or potential metal transporters), as well as *NASs*, *FROs* and *NAC5* (involved in metal homeostasis) in flag leaves of eight rice cultivars (showing contrasting levels of seed Fe and Zn) during panicle emergence (R3) and grain filling stage (R5). The expression level of nine of these genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) in flag leaves exhibited significant correlations with Fe and/or Zn concentrations in the seeds. In this way, our study has provided a short list of putative target genes to manipulate Fe and Zn concentrations in rice grains.
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Introduction

Rice is the primary or secondary staple food for 50% of the world's population. Therefore, it is one of the most important crop plants on Earth (Lucca et al., 2002). However, rice is a poor source of essential micronutrients such as Fe and Zn (Bouis and Welch, 2010). Micronutrient malnutrition, and particularly Fe and Zn deficiencies, affect over three billion people worldwide, mostly in developing countries (Welch and Graham, 2004). Biofortification has emerged as one possible solution to alleviate malnutrition and the development of new cultivars with elevated concentrations of Fe and Zn would be extremely relevant (Zimmermann and Hurrell, 2002). However, the lack of knowledge about how nutrients are translocated from vegetative tissues to the seeds is one of the barriers to biofortification (Colangelo and Guerinot, 2006), resulting in uncertainty about the best genes or pathways to target for modification. In this way, a better understanding of metal homeostasis and localization, as well as the identification of the molecular players that might contribute to the process of metal transport to the seeds is essential.

Around 75% of total grain Zn was reported to be present in the endosperm of brown rice (Jiang et al., 2008), while X-ray fluorescence imaging revealed that Zn is most abundant in the embryo and in the aleurone layer (Takahashi et al., 2009). Fe has been localized by histochemical techniques in the aleurone layer and in the embryo, but not in the endosperm of non-transformed rice seeds (Prom-u-thai et al., 2003; Sivaprakash et al., 2006; Sellappan et al., 2009). X-ray fluorescence imaging allowed the identification of Fe also in the endosperm (Takahashi et al., 2009). Although reported to be present in protein bodies of aleurone cells and in phytin granules both in the aleurone and in the embryo's scutellar cells (Krishnan et al., 2001), there is no clear information in the literature about the prevalent form of Fe (ferric or ferrous) present in the rice seed. In this work, we used staining techniques to investigate the distribution and relative abundance of both Fe forms in a set of genotypes, trying to identify specific distribution patterns characteristic of those genotypes with higher seed Fe concentrations.

Several transporters potentially involved in metal ion homeostasis have been identified in the rice genome (Bughio et al., 2002; Gross et al., 2003; Ramesh et al., 2003; Koike et al., 2004). Most of

Abbreviations: DMA, deoxymugineic acid; FRO, ferric reductase oxidase; ICP-OES, inductively coupled plasma optical emission spectroscopy; IRT, iron-regulated transporter; NA, nicotianamine; NAC, NAC transcription factor; NAS, nicotianamine synthase; NRAMP, natural resistance-associated macrophage protein; VIT, vacuolar iron transporter; YSL, yellow stripe-like; ZIP, Zrt/Irt-like protein.

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these metal transporters are capable of transporting one or several divalent cations including Fe^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} (Narayanan et al., 2007). However, most of these genes belong to gene families and the specific function of several of these transporters remains unknown. Furthermore, little information is available about how metals are transported to rice seeds and which of the processes (uptake from the soil, transport from the roots to the shoots, phloem loading, grain filling) are the rate-limiting steps for metals to reach the grain. This information, together with the identification of the genes which actively participate in each one of these processes, is essential to develop rice cultivars with improved nutrient concentrations.

Flag leaves are the major source of phloem-delivered photoassimilates for rice developing seeds, and are believed to be one of the sources of remobilized metals for the seeds (Narayanan et al., 2007; Sperotto et al., 2009). In another cereal (wheat), Zn and Fe remobilization from flag leaves to seeds was clearly demonstrated (Uauy et al., 2006; Waters et al., 2009). Our group has previously shown that flag leaf Zn concentration decreases during reproductive development in rice cultivars with high seed Zn concentrations (Sperotto et al., 2009). In the same work, flag leaf Fe concentrations decreased during reproductive development in a rice cultivar with high Fe concentration in seeds, while a cultivar with low seed Fe concentration showed a high level of residual Fe in flag leaves at reproductive maturity. Another group has presented evidence for net Zn remobilization from rice leaves and transport to the grain when the plants were challenged with reduced levels of root-supplied Zn during grain fill (Jiang et al., 2008). Together, these studies support the concept that at least some of the rice grain's Zn and Fe are mobilized from flag leaves and suggest that the relative contribution of the flag leaf may vary across different genotypes.

It is possible that other mechanisms of mineral allocation to the rice grain, such as remobilization from other leaves, phloem delivery of minerals acquired from the xylem through a transfer process or even direct xylem loading, may contribute with higher Fe and Zn amounts than flag leaf remobilization during grain filling in rice. However, significant increases in flag leaf remobilization could have a positive impact in biofortification efforts. Enhanced flag leaf remobilization could be achieved in the future, provided that the relevant genes and gene products are identified and studied. As we were interested in identifying genes that could be targets for improved Fe and Zn mobilization to seeds, we analyzed gene expression of 25 metal-related genes in flag leaves of eight different rice cultivars (showing contrasting levels of Fe and Zn in the seeds) during the period of panicle emergence and grain filling, which correspond to R3 and R5 stage, according to Counce et al. (2000). Furthermore, we correlated the expression level of these genes with final Fe and Zn concentrations in the seeds and were able to detect putative target genes to be used in conventional breeding or plant biotechnology to improve Fe and/or Zn concentrations in rice grains.

Materials and methods

Plant growth conditions

Plants from eight rice (*Oryza sativa* L.) cultivars [Canastra, BR-IRGA421, BR-IRGA409, IR68144 and IR68144_1 (five *indica* cultivars) and IR75862_1, IR75862 and IR69428 (three *japonica* cultivars)] were grown in soil under flooded conditions in the experimental unit of Instituto Rio-Grandense do Arroz (IRGA), in Cachoeirinha, RS, Brazil (29°54′58.61″S, 51°10′02.65″W), during the rice growing season (October 2007 to March 2008). Soil characteristics of this site were reported by Stein et al. (2009a). Entire flag leaf blades were collected during R3 (panicle emergence) and R5 (grain filling) stages (Counce et al., 2000), and immediately frozen in liquid nitrogen. Three samples were collected from a plant with multiple tillers and samples were pooled. Three biological replicates (independent plants) were collected for quantitative PCR studies. Seeds were also harvested from plants grown to full maturity (R9, according to Counce et al., 2000) for histochemical localization of Fe forms and for Fe and Zn quantification.

Iron localization in rice grains

Presence and localization of Fe was determined on rice grains of six genotypes (Canastra, BR-IRGA421, BR-IRGA409, IR68144_1, IR75862 and IR69428) with Prussian's and Turnbull's Blue histochemical reactions (Lillie, 1965). Prussian Blue reaction detects ferric iron (Fe^{3+}) through the treatment of samples with potassium ferrocyanide [$K_4Fe(CN)_6{\cdot}3H_2O$] acid solution (100 mg of ferrocyanide diluted in 10 ml of HCl 0.06 M), while Turnbull's Blue reaction localizes Fe in the ferrous form (Fe^{2+}) by exposing the samples to potassium ferricyanide [$K_3Fe(CN)_6$] acid solution (100 mg of ferricyanide diluted in 10 ml of HCl 0.06 M). Iron molecules present in the tissue result in a blue pigment in both reactions (Lillie, 1965). Grains of the six cultivars were exposed to 60 °C overnight to avoid germination and then soaked for 12 h in distilled water for free-hand sectioning. Grains were sectioned in half before being exposed to the solutions. Samples (14 half seeds per genotype) were kept in the solution for a minimum time of 1 h and a maximum of 1 h and 30 min for both reactions. The aleurone layer was evaluated on the external view, while endosperm, embryo and subaleurone layer were evaluated in the longitudinal internal section of the grain. Observations of rice grains were made in a Wild Heerbrugg stereoscopic microscope and documentation with a Kodak C-140 under bright-field mode.

Fe and Zn quantification by ICP

Fe and Zn concentrations were determined on rice grains of eight genotypes (Canastra, BR-IRGA421, IR68144, BR-IRGA409, IR75862_1, IR75862, IR68144_1 and IR69428). Whole seeds from nine plants were harvested and dried in a 60 °C oven for 48 h. Dried tissues were digested according to Narayanan et al. (2007). Fe and Zn quantification was performed using inductively coupled plasma optical emission spectroscopy (ICP-OES - CIROS ICP Model FCE12; Spectro, Kleve, Germany). Tomato leaves and rice flour standards (SRM 1573A and 1568A, respectively; National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the rice samples to ensure accuracy of the instrument calibration.

RNA extraction and cDNA synthesis

Total RNA was extracted using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Mountain View, CA, USA), according to the manufacturer's instructions, in the presence of RNase OUT (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA, USA), using 1 μg of RNA.

Quantitative RT-PCR and data analysis

qRT-PCRs were carried out in an Applied-Biosystem 7500 real-time cycler. All primers (listed in Supplementary Table 1) were designed to have similar T_m values (60 ± 2 °C). Reaction settings were performed according to Sperotto et al. (2009). Gene expression was evaluated by the $2^{-\Delta C_T}$ method (Livak and Schmittgen,

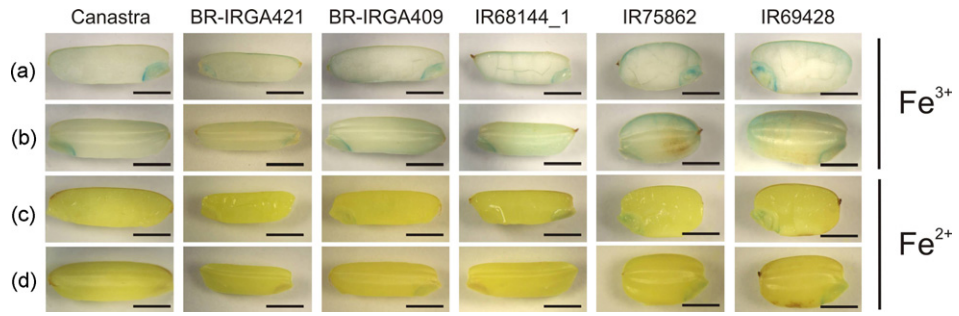

Fig. 1. Histochemical localization of ferric Fe through Prussian's reactions (a and b) and of ferrous Fe through Turnbull's Blue reactions (c and d) in six rice cultivars. External view of rice grains (b and d) and longitudinal internal sections (a and c) are shown. Representative images of 14 replicates per genotype and stained solution are shown. Bars = 200 μm.

2001; Schmittgen and Livak, 2008). For each sample, analyzed in four technical replications, a ΔC_T value was obtained by subtracting the Ubiquitin C_T value from the C_T obtained for the gene of interest. Each data point corresponds to three true biological replicate samples.

Statistical analyses

When appropriate, data were subjected to analyses of variance (ANOVA) and means were compared by the Tukey HSD (Honestly Significant Differences) test ($P \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., USA). The Levene's test (for homogeneity of variance) was used prior to ANOVA. Pearson's correlation analyses were carried out using two significance levels ($P \leq 0.05$ and 0.01).

Results and discussion

Fe distribution in grains of different rice cultivars

Through the use of histochemical methods, no Fe in either the ferrous or ferric form was detected in endosperm of the evaluated genotypes. Ferrous Fe was observed in the embryo of only two genotypes: IR75862 and IR69428 (Fig. 1c and d) and was not detected in the aleurone and subaleurone layer. Fe in the ferric form was seen in all genotypes (Fig. 1a and b). Low amounts of ferric Fe were detected in the aleurone layer. For the genotypes which seem to have Fe in the aleurone layer in Fig. 1b images, further analysis of the sections shown in Fig. 1a indicate that the blue color is in fact the reaction present in the subaleurone layer, seen visibly through the aleurone layer. Ferric Fe was visible in the subaleurone layer of BR-IRGA421, BR-IRGA409, IR68144_1, IR75862 and IR69428 (Fig. 1a and b). The subaleurone layer consists of the endosperm's two outermost cell layers, which are known to be rich in proteins and lipids and to have smaller amyloplasts and compound starch granules than the inner endosperm (Juliano, 1993). It is possible to see the reaction in the subaleurone layers, in the external view, due to the fact that the aleurone is generally formed by one layer of cells (Sabelli and Larkins, 2009).

Ferric Fe could be seen in the embryos of all genotypes (Fig. 1a and b). However, the responses to the reaction varied between genotypes, probably due to different Fe content in the tissue. IR69428 was one of the genotypes with the strongest reaction to the potassium ferrocyanide solution, indicating that this genotype's grain has one of the highest Fe concentrations among those evaluated.

Ferric iron, the prevalent Fe form in the rice grain according to our results, could be present in phytin granules, where phytin salts of potassium and magnesium also contain other mineral cations such as Fe^{3+} (Inoue et al., 2009 and references within). Ferric Fe could also be stored in the central cavity of the ferritin protein. However, rice ferritins are probably more involved with oxidative stress protection than with iron storage in the grain (Stein et al., 2009b), similar to *Arabidopsis*, where no more than 5% of total seed Fe is stored in ferritin (Ravet et al., 2009). It was also recently demonstrated that iron and ferritin accumulate in separate cellular locations in *Phaseolus* seeds (Cvitanich et al., 2010).

It would be possible to perform semi-quantitative analysis of Fe using the images obtained (Choi et al., 2007). However, we decided to use a more precise technique, ICP-OES. Moreover, this technique allowed us to evaluate the concentration of Zn, another important micronutrient. Considering that we planned to perform correlation analyses between metal concentrations and gene expression, two more genotypes (IR68144 and IR75862_1) were included in the subsequent analyses.

Fe and Zn concentrations in seeds

Elemental analyses were performed on mature seeds (R9 stage) of eight rice cultivars by ICP-OES (inductively coupled plasma optical emission spectroscopy). Canastra plants were found to have a significantly lower seed Fe concentration ($P \leq 0.05$) relative to all the other cultivars (Table 1), being approximately 45% lower than the highest seed Fe concentrations found in the IR75862, IR68144_1 and IR69428 cultivars. These results corroborate our histochemical data, where IR75862 and IR69428 cultivars showed the presence of ferrous Fe in the embryo (Fig. 1c and d) and IR75862, IR68144_1 and IR69428 cultivars indicated stronger staining of ferric Fe in the subaleurone layer (Fig. 1a and b). Seed Zn concentration was higher in IR75862_1, IR75862, IR68144_1 and IR69428 than in the other four cultivars (Table 1). In order to find the relationship between Fe and Zn concentration in seeds, Pearson's correlation analyses were performed and indicated a positive correlation (0.620, $P = 0.0035$), suggesting that higher Fe and Zn concentrations may occur simultaneously in rice. Positive correlation between seed Fe and Zn concentrations was previously reported in wheat (Cakmak et al., 2004; Morgounov et al., 2007) and rice (Jiang et al., 2007; Sperotto

Table 1
Fe and Zn concentrations in whole seeds from eight diverse rice cultivars. Seeds were collected at full maturity (R9 stage). Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). DW = dry weight.

	Fe (μg g^{-1} DW)	Zn (μg g^{-1} DW)
Canastra	9.83 ± 0.14 c	22.36 ± 0.62 b
BR-IRGA421	12.47 ± 0.24 b	23.59 ± 0.48 b
IR68144	15.11 ± 1.30 ab	21.91 ± 1.05 b
BR-IRGA409	15.22 ± 1.13 ab	22.13 ± 0.25 b
IR75862_1	16.53 ± 0.43 a	34.84 ± 0.75 a
IR75862	17.27 ± 1.25 a	31.07 ± 0.53 a
IR68144_1	17.65 ± 0.60 a	30.73 ± 0.30 a
IR69428	18.74 ± 0.17 a	28.66 ± 0.51 a

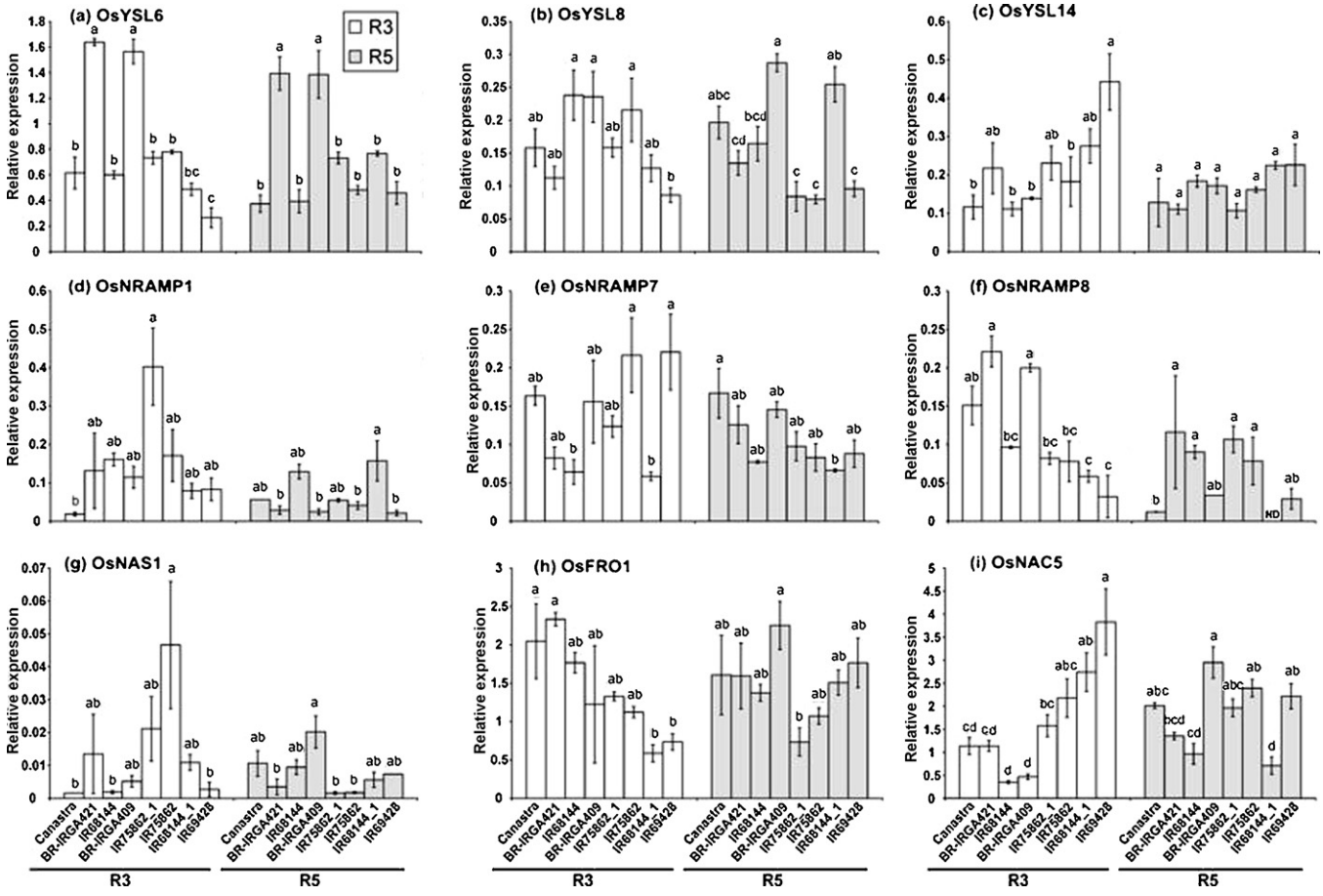

Fig. 2. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of (a) *OsYSL6*, (b) *OsYSL8*, (c) *OsYSL14*, (d) *OsNRAMP1*, (e) *OsNRAMP7*, (f) *OsNRAMP8*, (g) *OsNAS1*, (h) *OsFRO1* and (i) *OsNAC5* genes in flag leaves of eight diverse rice genotypes collected in R3 (panicle emergence) and R5 (grain filling) stages. Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). ND = not detected. Relative expression levels of tested genes with no statistically significant correlation with Fe and Zn concentrations in whole seeds are shown in Supplementary Figs. 1–4.

et al., 2009). The variation among genotypes was higher in seed Fe concentrations than in Zn concentrations (Table 1). Higher variations in Fe than in Zn concentrations were previously reported (Rengel et al., 1999), including a survey in which Fe and Zn concentrations among genotypes varied 13-fold and 1.2-fold, respectively (Marr et al., 1995).

Gene expression analyses in flag leaves

The expression patterns of 25 Fe and/or Zn-homeostasis related genes (nine genes from the YSL family of metal-phytosiderophore transporters: *OsYSL2*, *OsYSL5*, *OsYSL6*, *OsYSL7*, *OsYSL8*, *OsYSL10*, *OsYSL14*, *OsYSL15* and *OsYSL18*; six genes from the NRAMP family of metal transporters: *OsNRAMP1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsNRAMP7* and *OsNRAMP8*; four genes from the ZIP family of divalent metal transporters: *OsZIP4*, *OsZIP5*, *OsZIP6* and *OsZIP7*; two genes from the NAS family of enzymes necessary for nicotianamine biosynthesis: *OsNAS1* and *OsNAS2*; one gene from the IRT family of Fe^{2+} transporters: *OsIRT1*; one gene of the FRO family of ferric-chelate reductase/oxidase protein: *OsFRO1*; one gene of the vacuolar iron transporter family: *OsVIT1* and one gene from the NAC family of transcription factors: *OsNAC5*) were analyzed in flag leaves of eight different rice cultivars (showing contrasting levels of Fe and Zn in the seeds) during two reproductive developmental stages: R3 (panicle emergence) and R5 (grain filling) (Fig. 2 and Supplementary Figs. 1–4). The locus ID numbers of the 25 analyzed genes, according to the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), are presented in Supplementary Table 2.

The expression pattern of the tested genes varied among the eight cultivars. Surprisingly, there were no genes with up-regulated expression in R5 flag leaves relative to R3 across all tested cultivars. When differences were seen, they usually occurred only in one to a few cultivars, and were not a consistent trend. Most of the genes were found to be expressed in flag leaves, and expression of *OsYSL6*, *OsFRO1* and *OsNAC5* genes reached higher levels than *OsUBQ* expression (Fig. 2). Other genes (*OsNAS1*, *OsNAS2*, *OsNRAMP4*, *OsNRAMP5*, *OsZIP4*, *OsZIP5* and *OsIRT1*) exhibited low to non-detectable expression in flag leaves (Fig. 2 and Supplementary Figs. 2–4). Expression of *OsNAC5*, *OsYSL10*, *OsYSL14*, *OsYSL15*, *OsYSL18*, *OsNRAMP5*, *OsZIP5*, *OsIRT1* and *OsVIT1* genes was high at the R3 stage of at least one of the highest Fe and Zn cultivars (IR75862 _1, IR75862, IR68144_1 and IR69428) (Fig. 2 and Supplementary Figs. 1–4). On the other hand, expression of *OsYSL6*, *OsYSL7*, *OsYSL8* and *OsNRAMP8* genes was low at the R3 stage in the highest Fe cultivar (IR69428) (Fig. 2 and Supplementary Fig. 1). There were no major differences in gene expression during the R5 stage among the eight tested cultivars.

Correlation between gene expression in flag leaves and Fe and Zn concentration in seeds

To search for relationships between gene expression in flag leaves during different stages of reproductive development (R3 and

Table 2
Correlation analyses between expression data of nine metal-related genes in flag leaves during the reproductive development (stages R3 and R5) and Fe and Zn concentrations in seeds from eight rice cultivars (Canastra, BR-IRGA421, BR-IRGA409, IR68144, IR75862_1, IR68144_1, IR75862 and IR69428). A complete list of all genes tested is shown in Supplementary Table 3.

Gene	Fe		Zn	
	R3	R5	R3	R5
OsYSL6	−0.322	−0.116	−0.400*	−0.209
OsYSL8	−0.109	−0.205	−0.307	−0.462*
OsYSL14	0.539**	−0.150	0.406*	−0.097
OsNRAMP1	0.244	0.152	0.518**	0.061
OsNRAMP7	0.084	−0.602**	0.139	−0.416*
OsNRAMP8	−0.606**	0.053	−0.605**	0.224
OsNAS1	0.199	−0.182	0.471*	−0.535**
OsFRO1	−0.709**	−0.109	−0.510**	−0.359
OsNAC5	0.512*	−0.035	0.528**	−0.064

* Significant at 0.05 probability level.
** Significant at 0.01 probability level.

R5) and final Fe and Zn concentrations in seeds, Pearson's correlation analyses were performed for eight different rice cultivars. Final seed Fe concentration was positively correlated with *OsYSL14* and *OsNAC5* expression in flag leaves during R3 and negatively correlated with *OsNRAMP8* and *OsFRO1* expression during R3 and with *OsNRAMP7* expression during R5 (Table 2). Final seed Zn concentration was positively correlated with *OsYSL14*, *OsNRAMP1*, *OsNAS1* and *OsNAC5* expression in flag leaves during R3 and negatively correlated with *OsYSL6*, *OsNRAMP8* and *OsFRO1* expression during R3 and with *OsYSL8*, *OsNRAMP7* and *OsNAS1* expression during R5 (Table 2).

Fig. 2 shows the expression profile of the genes for which we found significant correlations between expression in flag leaves during either R3 or R5 and final seed Fe or Zn concentration. The bars representing the different cultivars are shown (from left to right) according to the ascending order of final Fe concentrations in the same cultivars. Some correlations can be clearly visualized, such as the negative correlations of *OsNRAMP8* (Fig. 2f) and *OsFRO1* (Fig. 2h) and the positive correlations of *OsYSL14* (Fig. 2c) and *OsNAC5* (Fig. 2i) expression with Fe during R3.

These analyses allowed us to assess which of these genes might contribute positively or negatively to the process of Fe or Zn transport from flag leaves to the seeds. However, we acknowledge that this process is very complex and that the 25 genes analyzed in our study represent only a small set of the molecular players that may help mobilize metals from flag leaves (and possibly non-flag leaves) to developing seeds.

The YSL family of transporters is believed to transport NA–metal chelates across plant cell membranes. Experimental evidence points to a role of the YSL proteins in the long-distance and intracellular transport of metals, especially Fe, complexed to NA (Curie et al., 2009; Ishimaru et al., 2010). Of the nine tested genes from the YSL family of metal–NA transporters, the expression levels of two (*OsYSL6* and *OsYSL8*) showed negative correlation with final Zn concentration in the seeds and one (*OsYSL14*) showed positive correlation with final Fe and Zn concentrations in the seeds (Table 2). None of these transporters has been functionally characterized. The *OsYSL14* gene product was previously suggested to be involved in the movement of metals within the plant, since the expression pattern of this gene is restricted to aerial plant organs (Nishizawa, 2006). The negative correlation found for *OsYSL6* and *OsYSL8* could be explained by the putative role of YSL proteins in metal movement to the vacuole. In this way, higher expression of such genes could result in higher proportion of Fe and Zn unavailable to be transported to seeds. Interestingly, AtYSL4 and AtYSL6 were identified in an *Arabidopsis* tonoplastic proteomic study (Jaquinod et al., 2007), and according to Curie et al. (2009), *AtYSL4* and *AtYSL6*, together with the two rice members *OsYSL5* and *OsYSL6*, form a separate cluster in the YSL family, suggesting that both rice proteins could also be located to the tonoplast and cause metal–NA complexes to flow out from the cytoplasm to the vacuolar compartment (Curie et al., 2009). If localized in the plasma membrane, the OsYSL5 and OsYSL6 proteins could be necessary for metal uptake into leaf cells with high metal demand, also decreasing metal availability for phloem loading.

In addition to the YSL genes, the expression level of the enzyme-coding gene *OsNAS1* in flag leaves during the R3 stage was positively correlated with and during the R5 stage was negatively correlated with Zn concentration in the seeds. NAS is required for the biosynthesis of NA, a non-peptidyl metal chelator that is believed to be a co-substrate of the YSL proteins (Roberts et al., 2004; Schaaf et al., 2004). Fe and Zn are potentially chelated by NA during phloem transport (von Wirén et al., 1999) and NAS genes are expressed in cells involved in long-distance transport of Fe (Inoue et al., 2003). Overexpression of barley *NAS* gene in tobacco increased Fe and Zn concentrations in young leaves, flowers and seeds of transgenic plants (Takahashi et al., 2003), indicating that NA promoted the transport of Fe and Zn to young leaves and reproductive organs in dicotyledonous plants.

The family of natural resistance-associated macrophage protein (NRAMP) metal ion transporters plays a major role in metal ion homeostasis in different species from bacteria to human (Nevo and Nelson, 2006). Of the six tested genes from the NRAMP family, one (*OsNRAMP1*) showed positive correlation with final Zn concentration in the seeds and two (*OsNRAMP7* and *OsNRAMP8*) showed negative correlation with final Fe and Zn concentrations in the seeds (Table 2). Several NRAMP proteins have been shown to transport multiple classes of cations, suggesting that NRAMP proteins may exhibit broad substrate specificity (Curie et al., 2000). OsNRAMP1 belongs to the same class as AtNRAMP1, which was shown to transport Fe (Curie et al., 2000). The subcellular localization of AtNRAMP3 on the vacuolar membrane suggests a function in intracellular metal homeostasis (Thomine et al., 2003). *OsNRAMP7* is the closest rice homolog to *AtNRAMP3* (data not shown). According to Stangoulis et al. (2007), there is a QTL for grain Fe concentration on chromosome 12 explaining approximately 14% of the phenotypic variation. Co-located with this QTL for Fe, there is a QTL for grain Zn concentration explaining approximately 13% of the phenotypic variation (Stangoulis et al., 2007; Garcia-Oliveira et al., 2009). A more in depth analysis revealed that *OsNRAMP7* locates inside this QTL (data not shown). However, it is still unknown whether NRAMP proteins drive metal influx or efflux from the vacuole. Based on our correlation analysis, OsNRAMP1 could function as a metal efflux transporter (participating in the export of metals from the vacuolar compartment to the cytosol), resulting in increased metal concentration available to be transported to the seeds. OsNRAMP7 and 8 could function as metal influx proteins (participating in the vacuolar sequestration of metals). However, the demonstration of this hypothesis requires further investigation.

In Strategy I plants, the reduction of Fe^{3+} is a prerequisite for the transport of Fe^{2+}, and Fe-deficiency leads to activation of Fe^{3+}-chelate reductase at the root surface. However, in rice, no Fe^{3+} reductase activity is detected at the root surface (Ishimaru et al., 2006). In leaves, *OsFRO1* is expressed under Zn-, Mn- and Cu-deficiency (Ishimaru et al., 2006). However, we were able to detect high expression of *OsFRO1* in flag leaves of rice plants grown under usual field conditions (Fig. 2), indicating that reduction steps may be needed for internal mineral transport. Our data show that *OsFRO1* expression in R3 flag leaves negatively correlates with Fe and Zn concentrations in seeds. According to Jeong et al. (2008), AtFRO7 plays a role in chloroplast Fe acquisition. Phylogenetic analyses show that *OsFRO1* and *AtFRO7* are closely related (Vinícius de Abreu Waldow, personal communication). In this way, OsFRO1

could have similar function in rice, directing Fe to the chloroplasts and avoiding its mobilization from flag leaves. A possible role for OsFRO1 in import of Fe to chloroplasts would not be unexpected given the importance of Fe for photosynthesis. In addition, it is thought that Fe storage in plants mainly occurs in plastids as the Fe storage protein, ferritin, is plastid-localized in plants (Zancani et al., 2007). It is already known that Fe^{3+} reduction is necessary for the subsequent uptake by ferritin (Laulhere and Briat, 1993). However, Fe^{3+} reduction is probably needed in a variety of other locations.

OsNAC5 has been recently characterized as a novel senescence-associated ABA-dependent NAC transcription factor (Sperotto et al., 2009). Higher and earlier *OsNAC5* expression is seen in flag leaves and panicles of IR75862 plants, which have higher seed Fe, Zn and protein concentrations than other tested genotypes (Sperotto et al., 2009). We found a positive correlation between the expression of this gene during the panicle emergence stage (R3) in flag leaves and final Fe and Zn concentrations in the grains. Lu et al. (2008) identified in rice a QTL (*qZn-11*) for grain Zn concentration on chromosome 11, between markers C794 and RG118, accounting for 18.61% of the phenotypic variation. A detailed analysis revealed that *OsNAC5* locates inside this QTL (data not shown). In wheat, the presence of the Gpc-B1 allele confers earlier flag leaf senescence and it was suggested that the Gpc-B1 locus is involved in more efficient remobilization of amino acids, Zn, Fe and Mn from leaves to grains, in addition to its effect on earlier senescence of the green tissues (Uauy et al., 2006). These effects are caused by the *NAM-B1* gene, located in the Gpc-B1 locus, which encodes a NAC domain-containing protein (Uauy et al., 2006; Waters et al., 2009). It is already known that a large number of metal transporter proteins are up-regulated during *Arabidopsis* leaf senescence (Van der Graaff et al., 2006). It is possible that OsNAC5 protein regulates similar transporter genes in rice and that these genes are needed for effective Fe and Zn remobilization.

Surprisingly, most of the correlations found between metal-related genes expression in flag leaves with final seed Fe and Zn concentrations (Table 2) were found during R3 (panicle emergence stage), instead of during R5 (grain filling stage). Mineral remobilization in early stages of reproductive growth has been demonstrated in wheat. Expression of *NAM-B1* is responsible for the earlier onset of flag leaf senescence, resulting in more efficient remobilization of protein, Zn, Fe and Mn from leaves to the grains (Uauy et al., 2006). *NAM-B1* expression is seen right after anthesis (Uauy et al., 2006), which in rice corresponds to R4 stage (Counce et al., 2000). Moreover, the highest seed Zn concentration following foliar Zn applications in wheat was found at the beginning of seed development (around 10 days after anthesis), suggesting that applying foliar Zn during early stages of wheat reproductive development could be an effective way of increasing seed Zn concentration through efficient flag leaf export to the grain (Ozturk et al., 2006). More studies are needed to evaluate if this critical period for increasing seed metal concentration in rice occurs not only after anthesis but also during the R3 stage.

It is possible that other mechanisms of mineral allocation to the rice grain, such as remobilization from other leaves, phloem delivery of minerals acquired from the xylem through a transfer process or even direct xylem loading, may contribute more Fe and Zn than that of flag leaf remobilization during grain filling in rice. However, flag leaf remobilization is one mechanism that could be enhanced in biofortification efforts, provided that the relevant genes and gene products are identified and their contributions to the process understood.

The lack of statistical correlation between gene expression in the flag leaves and metal concentration in the seeds does not mean that such gene/gene products have no function in the process of Fe and/or Zn transport to distant sinks, such as seeds. A study with a higher number of cultivars and/or with a different set of genes could reveal other significant correlations. It is also important to consider that changes in mRNA levels may not correlate with changes in protein or enzyme activity, due to post-transcriptional processes. Nevertheless, the expression profiles defined in this study do provide a useful starting point for more in depth analyses of the molecular mechanisms behind Fe and Zn remobilization from flag leaves to rice seeds. Functional analyses of selected gene products, using yeast complementation studies to verify their transport capacities should be done to confirm their involvement with this process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.05.003.

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